STUDIES ON THE METABOLISM OF CYCLIC NUCLEOTIDES BY THE TOAD BLADDER: THE METABOLIC PRODUCTS OF cAMP

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Received 10 May 1973

1. Introduction

Since the discovery by Orloff and Handler [1] that the effects of vasopressin on toad bladder transporting epithelium were mediated by 3′5′ cyclic AMP (cAMP), it has been of importance to investigate the metabolism of adenosine compounds in toad bladder mucosal cells. Gulyassy [2] has published an initial study in this field and this paper confirms and extends his findings.

2. Materials and methods

Toads (*Bufo marinus*) were obtained from Mogul-ED of Oshkosh, Wisc., being originally of South American origin. They were kept on damp peat at 30°C until use. Radioactive compounds were obtained from the Radiochemical Centre Amersham, Bucks; cAMP and cyclic 3′5′ inosine monophosphate (cIMP) from Boehringer, Mannheim, GmbH. The remaining analytical grade chemicals were obtained from BDH Limited, Poole, Dorset.

Homogenisation and subcellular fractionation: Urinary bladders were removed from pithed toads, mucus and blood removed, and the mucosal cell layer scraped off with a glass microscope slide. Crude homogenates were produced in a pre-cooled glass hand homogeniser. For subcellular fractionation cells were suspended in 0.2 M sucrose containing 100 mM Tris–HCl, pH 7.4 and 10 mM KCl. Tubes were kept in ice and homogenised in an Ultra-Turrax (T P 18/2N Janke and Kunkel) homogeniser. Centrifugation was performed in MSE refrigerated centrifuges at 1000 *g* for 5 min, 9000 *g* for 15 min, 20 000 *g* for 30 min, 105 000 *g* for 1 hr and finally 350 000 *g* for 90 min.

Incubations for enzyme assays were performed at room temp. (20°C) and the reactions terminated by immersing the tubes in boiling water for 3 min. The methods of initial purification were as follows:

i) A modification of the barium precipitation method of Krishna et al. [3]. The incubation medium contained 1.6 mM ATP, 5 mM $MgCl_2$, 10 mM theophylline, 0.1% albumin, 25 mM Tris–HCl pH 7.4. 0.05 ml of this medium was incubated with 0.05 ml of homogenate. Incubations were performed with ^{14}C-labelled ATP, and the reaction terminated in boiling water. An equal volume of a solution containing 40 mM ATP and 12.5 mM cAMP was added, then 0.4 ml distilled water and 0.2 ml of 0.3 N barium hydroxide and 0.2 ml of 5% zinc sulphate were added, the relative concentrations being arranged so that the pH was between 7.5 and 8.0 Barium sulphate was spun down, and the precipitation repeated twice. The radioactivity remaining in the supernatant was counted by liquid scintillation spectrometry. Chromatography revealed that the radioactive products in the supernatant were largely inosine with insignificant amounts of cAMP and adenosine. Barium causes non-enzymatic conversion of ATP to cAMP, though in Krishna's original method this problem was reduced by an initial chromatography step which removes most of the ATP.

ii) Aluminium oxide columns [4]: After incubation of homogenates protein and non-cyclic nucleotides were removed by running through 0.4 × 8 cm columns of chromatographic grade aluminium oxide. The sample was washed through with 3 × 2 ml vol of distilled water. Recovery of [^{3}H]cAMP from the columns was

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90% and spectrophotometric estimates of recovery of cIMP indicate that this is of the same order. Samples were then evaporated to dryness in a stream of air and taken up in 0.05 ml 10% isopropanol and applied to paper chromatograms.

It was found later that deep-freezing the incubates enabled denatured protein and mucus to be removed by centrifugation in sufficient amounts to enable the samples to be subjected to paper chromatography without further purification. Paper chromatography was performed using Whatman No. 1 (46 × 57 cm) paper. Descending chromatography only was used. Solvents used will be described in the descriptions of individual experiments. The following types of chromatograms were run: two dimensional descending chromatograms, double chromatography by cutting out nucleotide-containing spots from an initial chromatogram and sewing these to a second paper to be re-run, 'over-run' chromatograms in which the solvent was allowed to drip off the serrated lower margin, and the position of nucleotides determined by running dye markers in parallel with nucleotide samples. Chromatograms were dried in a stream of warm air, nucleotide spots visualised by ultraviolet light, the spots cut out and radioactivity measured by liquid scintillation spectrometry.

Liquid scintillation spectrometry was performed in a Packard TriCarb Model 3320. Efficiency was estimated using either an external standard, or in the case of tritium, a channels ratio method [5]. Double isotope counting was done using calibration curves constructed using Nuclear Chicago ^{14}C and ^{3}H standards.

3. Results

Preliminary experiments confirmed the finding of Gulyassy [2] that large amounts of inosine are formed from ATP by toad bladder mucosal cell homgenates. These experiments were performed using broken cell preparations and ^{14}C-labelled ATP as a substrate. Tubes were incubated for 15 min at 20°C, the reactions terminated by immersion in boiling water for 3 min. Initial purification was performed using the barium precipition method of Krishna et al. [3]. The unprecipitated products of the reaction were separated by paper chromatography, and shown to be about 90% inosine with small amounts of adenosine, hypoxanthine and cAMP. Under the conditions of these experiments inosine was produced in quantities proportional to both time and ATP concentration. Inosine as a major reaction product was identified as follows:

i) Chromatographically: Paper chromatography in ethanol: 1.0 M ammonium acetate:water, 5:1:1 [6] the metabolic product co-chromatographed with authentic inosine. In this system hypoxanthine co-chromatographs with inosine.

ii) The product did not move from the origin when subjected to electrophoresis [7] in pyridine buffer at pH 3.5 by Dr. John Hindley, of the University of Bristol, Department of Biochemistry. It was resistant to alkaline hydrolysis and DNAase. It was therefore not a polynucleotide, but a small uncharged molecule.

iii) Mucosal cell homogenates were incubated with [^{32}P] ATP or [^{14}C] ATP under the conditions of the first experiments, and run on a paper chromatogram in ethanol—1 M ammonium acetate—water. Autoradiography of the paper revealed radioactivity from the [^{14}C] ATP not from that labelled with phosphate.

iv) The reaction product co-chromatographed with inosine in the following paper chromatographic system: isopropanol : concentrated ammonium hydroxide:water, 7:1:2 [8] and saturated ammonium sulphate:water:isopropanol, 79:19:2 [9]. By these methods the metabolic product was distinguished from hypoxanthine, adenosine, guanosine, adenine and sodium urate.

Cyclic AMP was also converted to inosine by mucosal cell homogenates. A broken cell preparation was incubated with [^{3}H] cAMP. The reaction was terminated in boiling water and 30 µl samples taken for paper chromatography without purification by aluminum oxide. The paper was run in ethanol: 1 M ammonium acetate:water, 5:1:1. The results are shown in table 1. These preliminary experiments led us to think that cIMP may be an intermediary in the conversion of cAMP to inosine, and therefore that there may be two alternative pathways of breakdown of cAMP, one via 5'AMP, the other via cIMP.

3.1. *Experiments on the production of cIMP from cAMP by toad bladder homogenates.*

The following incubation medium was used: 1 mM ATP, 2 mM $MgSO_4$, 3 mM theophylline and 5 mM potassium phosphate buffer, pH 7.2. Mucosal cells

Table 1
Metabolic products of [^{3}H]cAMP produced by toad bladder mucosal cell homogenates, separated by paper chromatography expressed as cpm.

Compound	Boiled control	Experimental	Differences
cAMP	119944	11120	−108824
ATP	142	1155	1013
5'AMP	229	576	347
Inosine	453	92213	91560
Adenosine	187	768	581

scraped from two toad bladders were homogenised in 1 ml of incubation medium. Each tube contained 1.4 ml medium, 100 μl homogenate and 250 μl [^{3}H] cAMP (0.5 mCi/ml), the incubates were purified using aluminium oxide, taken up in isopropanol and run on paper chromatograms in n-propanol: conc. ammonium hydroxide: water (60:30:10) [10]. The resulting nucleotide spots were cut out and divided into two, one half of each was first incubated with phosphodiesterase for 10 min at 37°C then both halves of each spot re-chromatographed. The radioactivity from the first chromatogram was: background 41 cpm, cIMP spot 15 056 cpm, and cGMP 3671. After re-running the products of phosphodiesterase incubation in the same solvent system the following counts were obtained: cIMP 197 cpm, 5′ IMP 6878, cGMP 192 cpm, 5′GMP 338 cpm. The same experiment was repeated and the products of the reaction run on paper in a two dimensional system: n-propanol: conc. ammonium hydroxide: water (60:30:10), in the first dimension, and saturated ammonium sulphate: water:isopropanol (79:19:2), counts per min were cIMP 80, in the boiled control, 9367 in the experimental sample. A further experiment in which the cIMP spot was cut out, sewn on another paper and rerun in ethanol: 1.0 M ammonium acetate:water (5:1:1) gave 88 cpm in the cIMP spot from the boiled control and 421 cpm from the experimental. In the final experiment the cIMP spot was cut out and incubated with phosphodiesterases for 2 hr and the resulting material re-run in the two dimensional system described above. The counts per minute in the 5′ IMP position were boiled control 204, and experimental 505.

4. Discussion

The experiments described confirm Gulyassy's findings that inosine is a major metabolic product of both ATP and cAMP [2]. They also indicate that cAMP can be converted to cIMP, and therefore suggest a possible alternative breakdown pathway for cAMP.

Another important finding is that the cAMP assay system using barium precipitation can lead to misleading results when ^{14}C-labelled ATP is used instead of the ^{32}P-labelled compound recommended by the authors [3]. The experiments give no indication of the fate of inosine in the metabolic pathways of toad bladder cells. The difficulties of measuring recoveries of metabolic products from paper chromatograms have precluded a rigorous quantitative study of the reactions outlined in both this and the following paper.

Acknowledgement

The authors are grateful for financial support from the Medical Research Council, London.

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